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# PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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<input type="checkbox"/> Additional inventors are being named on the _____ separately numbered sheets attached hereto					
TITLE OF THE INVENTION (280 characters max)					
ACE INHIBITORY PEPTIDES FROM PLANT MATERIALS					
Direct all correspondence to: CORRESPONDENCE ADDRESS					
<input checked="" type="checkbox"/> Customer Number <span style="border: 1px solid black; padding: 2px;">24223</span>		<div style="border: 1px solid black; padding: 5px; text-align: center;">             Place Customer Number Bar Code Label here  <span style="font-size: 1.2em;">24223</span> </div>			
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ENCLOSED APPLICATION PARTS (check all that apply)					
<input checked="" type="checkbox"/> Specification Number of Pages		<span style="border: 1px solid black; padding: 2px;">24</span>		<input type="checkbox"/> CD(s), Number	
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<input checked="" type="checkbox"/> Application Data Sheet. See 37 CFR 1.76					
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)					
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Respectfully submitted,

SIGNATURE

Patricia A. Rae

Date

Dec. 23/02

TYPED or PRINTED NAME

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REGISTRATION NO.

33570

(if appropriate)

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9777-16/PAR

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416-595-1155

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## **ACE INHIBITORY PEPTIDES FROM PLANT MATERIALS**

### **Field of the Invention**

This invention relates to improved processes for obtaining ACE inhibitory peptide  
5 compositions with high specific activity from plant sources.

### **Background of the Invention**

Hypertension, or high blood pressure, is a common health problem; in North America,  
one in four adults has hypertension. Since hypertension is asymptomatic, irreversible  
10 cardiovascular complications may have arisen before the hypertension is even recognised.

One enzyme which plays a key physiological role in the regulation of blood pressure is  
angiotensin converting enzyme (ACE: peptidyl dipeptide hydrolase, EC 3,4,15,1). ACE is  
involved in increasing blood pressure both through the renin-angiotensin-system, by virtue of its  
ability to convert the inactive decapeptide angiotensin I to the vasoconstrictive and salt-retaining  
15 peptide angiotensin II (Skeggs et al., 1956) and through the kallikrein-kinin system, by virtue of  
its ability to inactivate the vasodilator and natriuretic nonapeptide, bradykinin (Yang et al.,  
1970).

Inhibition of ACE activity therefore provides a means of lowering blood pressure.

The first potent and specific inhibitors of ACE activity were discovered by Ferreira  
20 (1965), who showed that an extract of the venom of the Brazilian arrowhead viper, Bothrops  
jararaca, potentiated smooth muscle contraction, caused hypotension and increased the capillary  
permeability induced by bradykinin. The so-called 'bradykinin potentiating factors' (BPF's)  
were later isolated from a number of snake venoms and found to be short peptides which are  
potent ACE inhibitors.

25 A class of synthetic ACE inhibitors has also been made and marketed commercially, the  
first being captopril (D-2-methyl-3-mercaptopropanoyl-L-proline). Although many drugs of this  
group have been found to be invaluable for lowering high blood pressure, their prolonged use  
can be accompanied by unwanted side effects. There therefore remains a need for new  
therapeutic agents to control hypertension.

30 It has been known for some time that ACE inhibitory peptides can be obtained by  
proteolytic digestion of proteins from various sources, including fish (eg. EP 1094071), animal  
milk proteins (eg. WO 99/65326) and plants (eg. Kawakami et al., (1995); Yano et al., (1996);

Pedroche et al., (2002); Wu et al., (2002)). Most of the methods described for obtaining ACE inhibitory peptides from plant proteins involve preliminary purification of the protein before hydrolysis, which adds to the cost and complexity of the process and therefore to the cost of the product.

There remains a need for improved processes for preparing ACE inhibitory compositions from plant materials and for further sources of ACE inhibitory peptides of high specific activity.

### **Summary of the Invention**

In accordance with one embodiment of the invention, a process for preparing an ACE inhibitory peptide-containing hydrolysate from a seed meal or a flour comprises contacting a substantially oil-free seed meal or flour with an organic solvent for an effective time period, separating the meal or flour from the solvent, subjecting the separated meal or flour to digestion by a proteolytic enzyme to generate ACE inhibitory peptides, and separating the digested meal or flour to obtain an ACE inhibitory peptide-containing hydrolysate.

The organic solvent may be selected from the group consisting of ethanol, methanol, butanol, acetone and ethyl acetate; ethanol is preferred, particularly 70% aqueous ethanol.

The seed meal or flour may be obtained from a plant such as flax, canola, soybean, cottonseed, sunflower, peanut mustard, pea, lentil, bean, chickpea, wheat, oats, barley, rye or buckwheat.

In accordance with a further embodiment, a process for preparing an ACE inhibitory peptide-containing hydrolysate from flax or canola comprises subjecting a substantially oil-free flax seed meal or a substantially oil-free canola meal to digestion by a proteolytic enzyme to generate ACE inhibitory peptides; and separating the digested meal to obtain an ACE inhibitory peptide-containing hydrolysate.

In accordance with a further embodiment is an ACE inhibitory peptide-containing hydrolysate produced by proteolytic digestion of a seed meal or flour, for example an ACE inhibitory peptide-containing hydrolysate produced by proteolytic digestion of flax meal or

canola meal. Also provided by the invention is a powder produced by drying a hydrolysate of the invention, for example by spray drying.

In accordance with a further embodiment is a food supplement comprising a hydrolysate of the invention, or a food or beverage product comprising a hydrolysate. Food and food supplements include a food supplement comprising an ACE inhibitory peptide-containing hydrolysate produced by a process of the invention, and a food or beverage product comprising an ACE inhibitory peptide-containing hydrolysate produced by a process of the invention.

In accordance with a further embodiment are the hypotensive peptides Val-Ser-Val and Phe-Leu, and hydrolysates containing these peptides.

In accordance with a further embodiment is a pharmaceutical composition comprising as active ingredient an agent selected from the group consisting of

- (a) a hydrolysate in accordance with the invention; and
- (b) at least one of peptides Val-Ser-Val and Phe-Leu.

### Summary of the Drawings

Certain embodiments of the invention are described, reference being made to the accompanying drawings, wherein:

Figure 1 shows ACE inhibitory activity (Y axis) as a function of incubation time (X axis), for various proteolytic enzymes acting on canola meal.

Figure 2 shows degree of hydrolysis (DH-Y axis) as a function of incubation time (X axis), for various proteolytic enzymes acting on canola meal.

Figure 3 shows absorbance at 214 nm (mAU) in fractions obtained from Sephasil Peptide C<sub>18</sub> 12 $\mu$  ST4.6/250 column.

### Detailed Description of the Invention

The present invention provides improved processes and new sources for preparing an ACE inhibitory composition from a plant material without the need to first isolate a highly enriched or purified protein fraction from the plant material.

The oilseeds flax and canola have not previously been shown to contain proteins which yield ACE inhibitory peptides on proteolytic hydrolysis.

In accordance with a first embodiment, the invention provides a process for preparing an ACE inhibitory peptide-containing hydrolysate from flax or canola meal by digesting the meal with a proteolytic enzyme to generate ACE inhibitory peptide-containing hydrolysates of high specific activity without further purification.

The optimum proteolytic enzyme is determined, and hydrolysis is carried out, as described herein.

In accordance with a further embodiment, the inventors have found that extracting a seed meal or flour with an organic solvent, prior to proteolytic digestion to produce ACE inhibitory peptides, provides hydrolysates with increased ACE inhibitory activity. By using the organic solvent extraction step, the inventors were able to overcome problems of low yield and low ACE-inhibitory activity which can be encountered when plant material is subjected to direct proteolytic digestion. Table 1 shows that for most plant materials tested, organic solvent extraction of the meal or flour prior to proteolytic digestion increased IC50 and protein content of the hydrolysate.

Suitable plant materials include seed meals and flours. As used herein, a "meal" means the non-oil portion of oilseeds after oil extraction, in ground form, and a "flour" means the ground seed of a non-oil producing plant such as a cereal or a legume. The processes of the invention may be applied to oilseed meals produced by conventional methods such as flaking or expelling an oil seed and defatting and solvent extraction of the resulting meal and to flours obtained from non-oil seeds, such as cereals, pseudocereals and legumes.

The plant material can be in the form of grits obtained by coarsely grinding the whole seed or defatted meal followed by screening and are classified as coarse (10-20 mesh), medium (20-40 mesh) or fine (40-80 mesh) grits according to particles size, or the plant material may be in the form of flour produced by grinding to very fine particles, so that 97% of the product passes through 100-mesh screen. The hull, husk and bran which are generally low in protein may be removed prior to production of the flour.

For oil seeds such as canola, flax and soybean, substantially oil-free seed meal is obtained by subjecting the seed to conventional defatting or oil-extraction methods such as expelling, expelling with solvent extraction and flaking and solvent extraction, as practiced in the commercial vegetable oil industry. The resulting meal usually contains less than 1% fat.

Seeds such as cereal and pseudocereal grains (eg. wheat, oats, rye, buckwheat) are naturally substantially oil free and do not require defatting.

The plant material to be processed by the method of the invention may be obtained from an oil-seed, such as flax, canola/rapeseed, soybean, cottonseed, sunflower, peanut or mustard, from a legume seed, such as soybeans, peas, lentils, beans or chickpeas or from a  
5 cereal grain such as wheat, oats, barley or rye or a pseudocereal such as buckwheat.

Preferred organic solvents are the lower alcohols such as methanol, ethanol, propanol and butanol, or acetone or ethyl acetate. Ethanol is preferred. The alcohols may be used alone or as an aqueous mixture containing at least 50% alcohol. 65% to 75% ethanol is  
10 preferred.

The organic solvent is preferably mixed with the seed meal or flour at a liquid: solid ratio in the range of about 8:1 to about 25:1, about 10:1 to about 20:1 being preferred.

The seed meal or flour is kept in contact with the organic solvent for about 1 hour up to about 24 hours, at a temperature from about 20°C to the boiling point of the organic  
15 solvent. Preferred conditions are 24 hours at room temperature (25°C), or a shorter time at a higher temperature, eg. 3 hours at 50°C. The meal/solvent slurry may be stirred or otherwise agitated during the contact period.

The treated meal or flour is then separated from the liquid solvent phase by any suitable conventional method, such as centrifugation, screening, filtration or decantation and  
20 may be washed with water to reduce the level of residual organic solvent. The material is then ready for proteolytic digestion to generate ACE inhibitory peptides. Alternatively, the residual organic solvent may be removed by hot air drying to produce a meal or flour that can be stored for future rewetting and proteolytic digestion.

Proteolytic digestion of the seed meal or flour, whether ethanol extracted or not, may  
25 be carried out generally by methods known to those of skill in the art and as further described in the examples herein.

The optimum choice of proteolytic enzyme for generation of ACE-inhibitory peptides may vary depending on the source of the protein to be digested. It is within the skill of one in the art to determine which proteolytic enzyme gives the highest IC50 in the hydrolysate for a  
30 particular plant material, as described in the examples and in Figure 1. It has been found, for example, that thermolysin is preferred for digestion of flax seed meal and soybean meal. Proteolysis is carried out for a period of time which yields the greatest ACE inhibitory IC50



in the hydrolysate. Generally, a digestion period of about 3 hours provides maximum inhibitory activity.

The proteolytic digestion is terminated by adjusting the pH of the reaction mixture away from the digestion pH, to give an ACE inhibitory peptide-containing hydrolysate. The hydrolysate is filtered or centrifuged, for example in a decanter centrifuge, to remove residual meal or flour. The filtered hydrolysate can be used as a food supplement or, preferably, is spray dried to give a water-soluble powder suitable for use as a food or food supplement or as a pharmaceutical. The use of protein and hydrolysed protein preparations as food supplements and their incorporation into food products is well known to those of skill in the food processing art.

The hydrolysates of the invention in liquid or powder form may be used to supplement beverages such as soft drinks, carbonated beverages, ready to mix beverages, milk and milk beverages and their derivatives, and foods such as sauces, condiments, salad dressings, fruit juices, syrups, desserts (e.g. puddings, gelatin, icings, and fillings, baked goods and frozen desserts such as ice creams and sherbets), soft frozen products (e.g. soft frozen creams, soft frozen ice creams and yogurts, soft frozen toppings such as dairy or non dairy whipped toppings), oils and emulsified products (e.g., shortening, margarine, mayonnaise, butter and salad dressings), candy and bar confections, cereal foods, and chewing gum tablet.

The hydrolysates may also be formulated as tablets, capsules, granules, powders, syrups, suspensions or injectable solutions.

The hydrolysates produced by the methods described herein have equivalent or greater ACE inhibitory activity than the commercially available whey hydrolysates. When analysed by the ACE inhibitory assay described herein, the commercial whey product BioZate ( Davisco Foods International) had an ACE inhibitory IC<sub>50</sub> of 137 µg powder/ml. As shown herein, several plant materials yielded protein hydrolysates with ACE inhibitory IC<sub>50</sub> values considerably greater than the whey product's inhibitory activity without the need to employ any subsequent purification strategy.

Proteolytic hydrolysates obtained by proteolytic digestion of canola meal were further purified, as described in Example 12, to give two fractions, each containing a single purified ACE-inhibitory peptide. These peptides, not previously described as ACE inhibitors, had the amino acid sequences Val-Ser-Val and Phe-Leu.

The ACE inhibitory peptide-containing hydrolysates of the invention may be further processed to obtain fractions with further enhanced specific activity or even the purified single peptides described above, for use as food supplements or as pharmaceuticals.

The ACE inhibitory activity of the hydrolysates of the invention may be further enhanced, if desired, by ultrafiltration as described in the Examples herein.

It has been found that seeds with a high polysaccharide content, such as flax and barley, give hydrolysates of high viscosity. Where the viscosity of the hydrolysate is greater than about  $10 \times 10^{-3}$  PaS (based on the viscosity of a 5 % solution in water at 25°C and a shear rate of 200 (1/s), ultrafiltration membranes up to pore size 100,000 MWCO can be used, but membranes with pore size up to 10000 are preferred.

For hydrolysates of lower viscosity, such as those obtained from canola, membranes of pore size up to 10000 MWCO may be used and membranes with pore size up to 3000 MWCO are preferred.

15

### EXAMPLES

The examples are described for the purposes of illustration and are not intended to limit the scope of the invention.

Methods of chemistry, molecular biology, protein and peptide biochemistry and immunology referred to but not explicitly described in this disclosure and examples are reported in the scientific literature and are well known to those skilled in the art.

#### **Reagents**

Chemicals and enzymes were obtained as follows: angiotensin converting enzyme (from rabbit lung) and HHL (Sigma Chemical Co., St. Louis, MO, USA); HA and trifluoroacetic acid (TFA) (Acros Organics, New Jersey, USA); HPLC grade acetonitrile (Fisher Scientific, Nepean, ON, Canada). All other chemicals were of reagent grade and also obtained from Fisher Scientific. HPLC-grade water was generated by a Milli-Q system (Millipore, Bedford, MA, USA).

30

## **Methods**

### **The determination of ACE-inhibitory activity**

The *in vitro* antihypertensive activity (ACE-inhibitory activity) was measured by a new improved HPLC method (Wu, J. P. et al., (2002)), modified from the method of Cushman and Cheung (1971). This method utilizes reverse phase high performance liquid chromatography (HPLC) to separate and quantify the ACE-catalyzed production of hippuric acid (HA). Hippuryl-histidyl-leucine (HHL) and HA were separated on a Symmetry C<sub>18</sub> column by gradient elution that used mixtures of trifluoroacetic acid (TFA)/acetonitrile and TFA/water as solvents. In comparison to the standard spectrophotometric method, the new HPLC method eliminates the need for ethyl acetate extraction of HA and allows direct injection of ACE reaction mixtures onto the HPLC column.

### **Sample preparation for HPLC analysis**

For direct HPLC analysis, the total reaction volume was 70 µl, composed of 50 µl of 2.17 mM HHL, 10 µl of 2 mU of ACE and 10 µl of different concentrations of protein hydrolysates (all prepared with 100 mM borate buffer, containing 300 mM NaCl, pH 8.3). For the control test, 10 µl of buffer solution was used. The HHL and protein hydrolysates or the control buffer solutions, were combined and maintained at 37°C for 10 min in 1.5 ml polyethylene micro-centrifuge tubes. ACE was also maintained at 37°C for 10 min before the two solutions were combined and incubated at 37°C in an Eppendorf Thermomixer R (Brinkmann Instruments, Inc. New York, USA) with continuous agitation at 450 rpm. The reaction was terminated after 30 min by addition of 85 µl of 1 N HCl and the solution filtered through a 0.45 µm nylon syringe filter for reverse phase RP-HPLC (RP-HPLC) analysis.

### **High-performance liquid chromatography**

HPLC was performed on a 2690 Separation Module equipped with a 996 Photodiode Array Detector (Waters Inc, MA. USA). Instrument control, data collection and analysis were undertaken using Millennium Chromatography Manager Software version 2.15 (Waters Inc, MA. USA). Samples (10 µl) were analyzed on a Symmetry C18 column (3.0 x 150 mm, 5 µm, Waters Inc., MA, USA) and HA and HHL were detected at 228 nm. The column was eluted (0.5 ml.min<sup>-1</sup>) with a two solvent system: (A) 0.05% trifluoroacetic acid (TFA) in

water and (B) 0.05% TFA in acetonitrile, with a 5-60 % acetonitrile gradient for the first 10 min, maintained for two min at 60% acetonitrile, then returned to 5% acetonitrile for one min. This was followed by isocratic elution for four min at the constant flow rate of 0.5 ml.min<sup>-1</sup>. External standard HA samples were prepared freshly and used for calculation of the concentration of HA formed by the action of ACE in the presence of protein hydrolysates (HA<sub>hydrolysate</sub>) or in the absence of protein hydrolysate (HA<sub>control</sub>), blank sample was prepared as: the ACE was inactivated first by the addition of HCl before the incubation.

#### ACE inhibitory activity calculation

Inhibitory activity was calculated according to the following equation:

$$\text{Inhibitory activity (\%)} = [ (HA_{\text{hydrolysate}} - HA_{\text{control}}) / (HA_{\text{control}} - HA_{\text{blank}}) ] \times 100$$

The IC<sub>50</sub> value was defined as the amount of inhibitory substance in the reaction mixture that resulted in 50% inhibition of ACE activity, measured as described above. A plot of inhibition activity (%) versus concentration of protein hydrolysate (µg powder/ml) was generated using at least 5 different concentrations of the same hydrolysate sample. Regression was conducted with the Microsoft Excel 97 SR-1 software (Microsoft Corporation).

#### Determination of Degree of Hydrolysis (DH) of Proteins

Under alkaline or neutral conditions, the pH was monitored and maintained constant by addition of base. The amount of base added was recorded at set time intervals and used to calculate the degree of hydrolysis (DH). The DH is calculated from the volume and molarity of base to maintain constant pH and is expressed as the percent ratio of numbers of peptide bonds broken (h) to the total numbers of bonds per unit weight according to the formula of Adler-Nissen (1986).

Under acid hydrolysis conditions, the degree of hydrolysis (DH) was determined according to the method of Adler-Nissen J. (1979).

#### Hydrolysate sample preparation

Samples of hydrolysis reaction mixtures were drawn at various time intervals and quickly transferred to test tubes. From each sample, 2.0 ml aliquots were rapidly pipetted into each of two test tubes containing 10 ml of 1% NaDodSO<sub>4</sub>, and the test tubes were kept at 75°C by immersing in a water bath with shaking for at least 15 min to disperse the protein hydrolysate.

The contents of each test tube were transferred quantitatively to a 50-ml volumetric flask and diluted to volume with 1% NaDodSO<sub>4</sub>. The content of free amino groups, expressed as leucine amino equivalents, was assayed by the TNBS reaction.

#### 10 TNBS Reaction

0.25 ml prepared sample or blank (1.0% SDS) or standard solution was mixed with 2.0 ml of phosphate buffer at pH 8.2.

2 ml of 0.1% TNBS solution was added and the test tube was shaken and placed in 50°C bath for 60 min, covered by aluminum foil.

4.00 ml of 0.1 N HCl was added to terminate the reaction and the absorbance was read against water at 340 nm after 30 min.

The DH was calculated using the modified method of Beak et al. (1995) as follows:

$$DH = (L_t - L_0) / (L_{\max} - L_0) \times 100$$

where  $L_t$  is the amount of  $\alpha$ -amino acid released at time  $t$ ;  $L_0$  is the amount of  $\alpha$ -amino acid in original canola solution; and  $L_{\max}$  is the maximum amount of  $\alpha$ -amino acid in canola meal after acid hydrolysis (conducted under 6N HCl at 100°C for 24 h).

#### Example 1. Enzymatic screening for the production of ACE inhibitory peptides (ACEIP) from defatted canola meal

The reaction was performed in batches in a reaction vessel equipped with a stirrer, thermometer, and pH electrode. Solvent extracted expeller cake commercial canola meal

(Canbra Foods, Lethbridge, Alberta, Canada), (31.16g), ground to pass a 40 mesh screen (protein content: 40.1%), was mixed thoroughly using a magnetic stirrer with distilled water to form 250 ml of a 5 % protein slurry. The proteolytic enzymes evaluated are listed in Table 2, along with their optimum pH and temperature conditions. The pH of the slurry was  
 5 adjusted to the appropriate pH and temperature for the enzyme being tested. Enzyme (4 %, w/w on the basis of protein content of slurry) was added to initiate the reaction. The pH of the reaction was maintained at a constant value by addition of either 0.5 N NaOH or 1 N HCl. The volumes of alkali were recorded at specific intervals for the calculation of degree of hydrolysis (DH).

10 Samples were taken at 30, 60, 120, 180, 300 min intervals and enzyme was inactivated by heating in boiling water for 15 min. The pH of the slurry was then adjusted to pH 4.0 to precipitate unhydrolysed proteins, large peptides and all solids. The precipitate was removed by centrifugation at  $10,000 \times g$  for 25 min. The resulting clear supernatants were then freeze dried and stored at  $-5^{\circ}\text{C}$  till further analysis. A control (without proteolytic  
 15 enzyme) sample was also generated using the same procedure. The freeze dried supernatants were then evaluated for their ACE inhibitory activity.

The ACE inhibitory activity (%) of canola protein hydrolysates digested by different enzymes is shown in Fig 1. Results indicated that ACE inhibitory activity (%) varied greatly, from 0 % to 50.5 %, with the average value of  $28.4 \pm 13.47$  %. Alcalase 2.4 produced the  
 20 most potent ACE inhibitory hydrolysate from canola meal. Other enzymes which generated significant ACE inhibitory activity included Protease S, Protease M, alkaline protease and neutral protease. Although not included in this study, Thermolysin was subsequently determined to produce a peptide fraction with similar ACE inhibitory activity to that observed for Alcalase 2.4 hydrolysates. Generally, any proteolytic enzyme which, in 3 hours  
 25 digestion, generates a hydrolysate giving  $>35$  % ACE inhibition is preferred. No ACE inhibitory activity was detected for the unhydrolysed control canola meal samples, indicating that it was the breakdown of canola protein by the action of proteolytic enzymes that resulted in the formation of ACE inhibitory peptides. When the incubation time for Alcalase 2.4 and Protease M was extended to 24 hours there was no significant increase in the ACE inhibitory  
 30 activity beyond 5 hours of incubation.

The relationship between degree of hydrolysis (DH) and incubation time is shown in Fig 2; DH increased with increased incubation time. Alkaline Protease and Alcalase 2.4 gave

the highest DH values. Protease M and Protease S enzyme treatments had moderate DH values, while their corresponding hydrolysates showed relatively high ACE inhibitory activity. Pepsin, Chymotrypsin and trypsin had the lowest DH values, which indicated that these enzymes had very limited ability to hydrolyse canola protein. There was no positive relationship between DH value and ACE inhibitory activity.

Sequential treatment of canola meal with different hydrolytic enzymes was investigated. After three hours of hydrolysis with Alcalase 2.4, the pH and temperature of the solution was adjusted to the optimum conditions for each of the four other enzymes that produced hydrolysates with high ACE inhibitory activity. The second enzyme was then added and the solution incubated for a further 3 hours. Of the protein hydrolysates resulting from sequential hydrolysis with two different enzymes, only the product derived from sequential hydrolysis with Alcalase 2.4 and Alkaline Protease had a significantly improved IC<sub>50</sub> value (40.0 µg powder/ml) over the activity of the single enzyme product (Alcalase 2.4 = 68.6 µg powder/ml), or any of the other proteases by themselves (55.7-78.6 µg powder/ml).

#### **Example 2. Preparation of flaxseed meal enzymatic hydrolysates**

The reaction was performed in batches in a reaction vessel as in Example 1. Solvent extracted expeller cake commercial flaxseed meal (CanAmara Foods, Atton, Manitoba, Alberta) (50 g), ground to pass a 40 mesh screen (protein content: 33.9%), was mixed thoroughly using a magnetic stirrer with distilled water into a 750 ml slurry. The pH of the slurry was adjusted to 8.0 and temperature to 60°C for Alcalase 2.4 enzyme hydrolysis. The enzyme was added at a ratio of 4 % (w/w, on the basis of protein content of slurry). During the reaction, the pH of the slurry was maintained at a constant value by addition of 0.5 N NaOH. The enzyme was inactivated by adjusting the pH to around 4.0 with 6 N HCl. Unhydrolysed proteins, large peptides and insoluble material were removed by centrifugation at 6,000 g for 25 min. The residue was resuspended in 250 ml water and centrifuged under the same conditions. The resulting clear supernatants were then combined, freeze dried and stored at -5°C till further analysis. At the same time, the control extract (without enzyme) was prepared under the same conditions. The hydrolysate yield was 53.54% (by weight) with a protein content of 39.9%, and an ACE inhibitory IC<sub>50</sub> of 64.3 µg powder/ml.

A range of proteolytic enzymes was then evaluated, as in Example 1; results with flax were similar to those found with canola meal.

**Example 3. Production of ACE inhibitory peptides from Soy flour.**

5        Nutrişoy 7B soy flour (87g, Archer Daniels Midland CO., Decatur, Illinois) was mixed thoroughly using a magnetic stirrer with distilled water into 600 ml slurry. The slurry was adjusted to pH 8.0 and 60°C for enzymatic hydrolysis by Alcalase 2.4 as in Example 2. Enzyme was added at the ratio of 4 % (w/w, on the basis of protein content of slurry). During the reaction, the pH of the slurry was maintained constant with 0.5 N NaOH. The  
10        enzyme was inactivated by adjusting the pH to around 4.0 with 6 N HCl. Unhydrolysed proteins and large peptides as well as other polymers were removed by centrifugation at 6,000 x g for 25 min. The residue was resuspended in 300 ml water and centrifuged using the same conditions. The resulting clear supernatants were then combined and freeze dried and stored at -5°C till further analysis. The hydrolysate produced by Alcalase 2.4 had an ACE  
15        inhibitory IC<sub>50</sub> of 126.3 µg powder/ml with a product yield of 82.8% W/W.

**Example 4. Production of ACE inhibitory peptides from pea flour and oat flour**

Pea flour and oat flour (Parrheim Foods, Saskatoon, Canada) were incubated with Alcalase 2.4 under the same conditions as described in Example 2. The oat flour hydrolysate  
20        had the weakest ACE inhibitory activity (1200 µg powder/ml) of all the plant materials tested. Pea flour had an ACE inhibitory activity of IC<sub>50</sub> 87.1 µg powder/ml.

**Example 5. Production of ACE inhibitory peptides from whole buckwheat meal**

Buckwheat was purchased from a local grocery store, ground and passed through a 40  
25        mesh screen. Samples of the meal were hydrolysed with alcalase 2.4 L or Thermoase, as described in Example 2. The protein hydrolysate produced by Thermoase had an ACE inhibitory IC<sub>50</sub> of 78.6 µg powder/ml with a product yield of 18 %, while the ACE inhibitory IC<sub>50</sub> of the Alcalase 2.4 L-treated hydrolysate was 174.3 µg powder/ml, with a product yield of 37.1 %.

**Example 6. Production of ethanol-treated canola meal ACE Inhibitory hydrolysate**



Defatted commercial canola meal as in Example 1 (31.2g), ground to pass a 40 mesh screen, was extracted with 70 % (v/v) ethanol:water solution at a ratio of 1:10 (weight/volume), with agitation for 12 hours at room temperature or at 50°C for 3 hours. The meal was recovered, washed with distilled water and subjected to hydrolysis by Alcalase 2.4, Protease M, Protease S, or a sequential treatment of Alcalase 2.4 and Alkaline Protease, for a period not exceeding 3 hr per enzyme (combined incubation for total 6 hours). The ethanol-treated canola meal enzymatic digests were then fractionated by centrifugation to produce a supernatant containing the hydrolyzed peptides and a precipitate which contained the unhydrolyzed proteins and residual meal. These hydrolysates show ACE inhibitory activity (IC<sub>50</sub>) from 40.0 µg powder/ml to 64.3 µg powder/ml (Table 3). The protein content of the hydrolysates produced from ethanol-treated meal was significantly increased, from 41.7% to 54.6-60.4 %, except for the hydrolysate resulting from incubation with Protease S where the protein content was only 28.7%.

**Table 3. ACE inhibitory IC<sub>50</sub> and protein content of ethanol-treated canola meal hydrolysates**

<i>Enzyme</i>	<i>IC<sub>50</sub> (µg powder/ml)</i>	<i>Protein (%)</i>	<i>Product Yield (%)</i>
Protease S	64.3	28.7	19.3
Protease M	40.0	56.4	32.8
Alcalase 2.4	68.6	60.4	38.6
Alcalase 2.4L+ Alkaline Protease	40.0	54.6	45.4

**Example 7. Preparation of ethanol-treated defatted flaxseed meal enzymatic hydrolysates**

Defatted flaxseed meal as in Example 2 (50 g), ground to pass a 40 mesh screen (protein content: 33.9%), was treated with 70% (v/v) ethanol: water solution (1/15. w/v) at 50°C for 3 hours. The extracted flaxseed meal was recovered by filtration and dispersed in water to form a 750ml water slurry using a magnetic stirrer. The production of enzymatic hydrolysate with Alcalase 2.4 was performed as described as in Example 2. The ethanol-

treated defatted flaxseed hydrolysate had a yield of 52.0% based on product weight, with a protein content of 45.5% and the ACE inhibitory IC<sub>50</sub> was 51.4 µg powder/ml.

Defatted flaxseed meal recovered after the extraction of lignan (SDG) as reported by Westcott & Muir (U.S. Pat. No 5,705,618), was also used for the production of ACE  
5 inhibitory hydrolysates. The protein hydrolysate generated by Alcalase 2.4 treatment of this material had a similar ACE inhibitory activity (IC<sub>50</sub> = 55.7 µg powder/ml), indicating that the recovery of the valuable phytochemical SDG from the meal does not interfere with the ability to produce biologically active peptides from the residual meal.

10 **Example 8. Production of ACE inhibitory peptides from ethanol-treated soy flour using Alcalase 2.4 and/or Thermolysin enzymes**

Nutrisoy 7B soy flour (87g, Archer Daniels Midland CO., Decatur, Illinois) was treated with 70% ethanol for 3 hours at 50°C. The filtered residue was mixed thoroughly using a magnetic stirrer with distilled water into 600 ml slurry. The slurry was adjusted to the  
15 appropriate pH and temperature for digestion by Alcalase Acalase 2.4 L or Thermolysin, as in Table 2. The enzyme was added at a ratio of 4 % (w/w, on the basis of protein content of slurry) for Alcalase 2.4 or at 1 % (w/w, on the basis of protein content of slurry) for Thermolysin. During the reaction, the pH of the slurry was maintained constant with 0.5 N NaOH if necessary. The enzyme was inactivated by adjusting the pH to around 4.0 with 6 N  
20 HCl. Unhydrolysed proteins and large peptides as well as other polymers were removed by centrifugation at 6,000 x g for 25 min. The residue was resuspended in 300 ml water and centrifuged using the same conditions. The resulting clear supernatants were then combined and freeze dried and stored at -5°C till further analysis. The hydrolysate produced by Thermolysin had an ACE inhibitory IC<sub>50</sub> of 42.9 µg powder/ml with a product yield of  
25 64.7%, while Alcalase 2.4 treatment resulted in a hydrolysate with an ACE inhibitory IC<sub>50</sub> of 118.1 µg powder/ml with a product yield of 55.7%.

**Example 9. Production of ACE inhibitory peptides from flaxseed using Thermolysin**

Defatted flaxseed meal as in Example 2 (50 g), ground to pass a 40 mesh screen  
30 (protein content: 33.9%) was mixed thoroughly using a magnetic stirrer with distilled water into a 750 ml slurry. The pH of the slurry was adjusted to the appropriate pH and temperature for the following enzymes: Thermolysin (39 units/mg solid), or incubation with Alcalase 2.4

for 3 hour and followed by incubation for a further 3 hours with Thermolysin. The enzyme was added at a ratio of 4 % (w/w, on the basis of protein content of slurry) for Alcalase 2.4 or 1 % (w/w, on the basis of protein content of slurry) for Thermolysin. After initiation of the reaction, the pH of the slurry was maintained at a constant value by addition of 0.5 N NaOH if necessary. The enzyme was inactivated by adjusting pH to around 4.0 with 6 N HCl.

Unhydrolysed proteins and large peptides as well as other polymers were removed by centrifugation at 6,000 ' g for 25 min. The residue was suspended again with 250 ml water and centrifuged using the same conditions. The resulting clear supernatants were combined, freeze dried and stored at -5°C till further analysis. The hydrolysate produced by

Thermolysin had an ACE inhibitory IC<sub>50</sub> of 37.1 µg powder/ml. The hydrolysate resulting from the sequential hydrolysis of Thermolysin and Alcalase 2.4 had an ACE inhibitory IC<sub>50</sub> of 34.2 µg powder/ml. Thermolysin is a more effective enzyme for the production of ACE inhibitory peptides from flaxseed compared to Alcalase 2.4 alone which produced a hydrolysate with an IC<sub>50</sub> of 64.3 mg powder/ml.

#### **Example 10. Ultrafiltration processing of Canola ACE Inhibitory peptides**

Defatted canola meal (62.3g) ground to pass a 40 mesh screen was treated with 70% ethanol (v/v), at ratio of 1:10, W/V). After filtration to remove the aqueous alcohol, the residue was further digested by Alcalase 2.4 to produce an ethanol-treated canola protein hydrolysate. The resulting protein hydrolysate was further purified by ultrafiltration over 10000, 3000, 1000 molecular weight cut off (MWCO) membranes. Ultrafiltration was conducted using an Amicon Ultrafiltration Cell Model 8200 (Amicon Division, W.R. Grace & Co., Beverly, USA) by applying Argon at a pressure of 35 PSI to the solution on the retentate side of the membrane. The peptide hydrolyzate was initially separated into retentate and permeate fractions using a 10000 MWCO membrane. The permeate from the 10000 membrane was then subjected to ultrafiltration over the 3000 MWCO membrane to generate a 3000 MWCO retentate and a 3000 MWCO permeate. The 3000 MWCO permeate was further fractionated by ultrafiltration over a 1000 MWCO membrane to produce a 1000 MWCO permeate and a 1000 MWCO retentate. A second set of experiments were conducted where the total protein hydrolysate was subjected to ultrafiltration over the 3000 MWCO and 1000 MWCO membrane without prior treatment over the 10000 MWCO membrane. Permeates and retentates were collected and freeze dried for ACE inhibitory IC<sub>50</sub>

determination. All permeates contained peptides with greater ACE inhibitory activity than the corresponding retentate fractions. The highest ACE inhibitory activity ( $IC_{50} = 25.7 \mu\text{g}$  powder/ml) was found in the permeate obtained by direct ultrafiltration of protein hydrolysate over the 1000 MWCO membrane (Table 4). Peptide fractions with high ACE inhibitory activity ( $IC_{50} = 30.0-31.4 \mu\text{g}$  powder/ml) were also obtained by ultrafiltration over the 3000 MWCO membrane, either directly or after ultrafiltration over a 10000 MWCO membrane. Since the 3000 MWCO permeate product yield was higher than that obtained with the 1000 MWCO membrane, there was little advantage in proceeding to the 1000 MWCO step. As membrane flux decreases with decreasing pore size, the optimum yield was obtained with the 3000 MWCO membrane.

**Table 4.** ACE inhibitory  $IC_{50}$ , protein content and yield of permeates and retentates resulting from the ultrafiltration treatment of canola hydrolysate

	<i>IC<sub>50</sub> (<math>\mu\text{g}</math> powder/ml)</i>	<i>Protein content (%)</i>	<i>Product Yield (%)</i>
<b>stepwise operation</b>			
10,000 permeate	37.1	59.3	73.5
retentate	54.3	54.0	20.4
3,000 permeate	30.0	56.3	70.5
retentate	50.0	66.2	28.6
1,000 permeate	30.0	46.9	43.5
retentate	31.4	68.5	47.0
<b>Individual operation</b>			
10,000 permeate	37.1	59.3	73.5
retentate	54.3	54.0	20.4
3,000 permeate	31.4	58.9	73.3
retentate	52.9	57.0	25.2
1,000 permeate	25.7	50.3	56.0
retentate	52.9	68.0	35.7

The liquid hydrolysates prepared in Examples 7 and 9 were further purified by ultrafiltration over 10000 and 100000 molecular weight cut off (MWCO) membranes as in Example 10. Ultrafiltration was conducted individually, both permeates and retentates were collected and freeze dried for ACE inhibitory IC<sub>50</sub> determination. As in the previous example, all retentates contain peptides with greater ACE inhibitory activity than those found in the retentate (Table 5). The viscosity of the flaxseed hydrolyste was much greater than that observed for canola protein hydrolysates, therefore it was impracticable to conduct ultrafiltration with membranes of 3000 and 1000 MWCO. When operating with 10 K and 100 K MWCO membranes, difficulties were encountered when conducting ultrafiltration due to the formation of a film on the surface of the membrane. The formation of this film can be avoided by; A) stirring and heating the solution to a temperature greater than 40°C; B) adjusting the pH to pH 3-4; or C) combination of A and B. Compared to the ACE inhibitory IC<sub>50</sub> value of the original flaxseed hydrolysate (64.2 µg powder/ml), no significant improvement was achieved in these ultrafiltration steps since, because of the large pore size of the 100K MWCO membranes, large peptides can also pass through the membrane into the permeate. In the case of the ethanol-treated hydrolysate, the permeates had improved IC<sub>50</sub> values of 30.0 and 35.7 µg powder/ml, compared to the original value at 51.4 µg powder/ml, indicating that the activity of the ACE inhibitory peptide fraction produced from the alcohol washed defatted flax meal can be improved by passage over either an 10K or a 100K MWCO membrane.

**Table 5.** Yield, protein content and ACE inhibitory IC<sub>50</sub> of defatted flax meal Alcalase 2.4 L hydrolysates subjected to ultrafiltration.

	<i>IC<sub>50</sub> (µg powder/ml)</i>	<i>Protein content (%)</i>	<i>Product Yield (%)</i>
<b>Defatted flaxseed hydrolysate</b>			
100K permeate	48.6	44.5	53.6
100K retentate	54.3	32.1	39.3
10K permeate	48.6	44.8	50.9
10K retenate	61.4	31.6	39.0

<b>EtOH treated defatted flaxseed hydrolysate</b>			
100K permeate	30.0	52.3	45.6
100K retentate	78.6	37.9	48.8
10K permeate	35.7	53.5	54.8
10K retenae	45.7	35.1	38.1

**Example 12. Purification and identification of highly bioactive ACE inhibitory peptides from canola protein hydrolysates by reversed phase chromatography**

Defatted canola meal Alcalase 2.4 hydrolysate prepared as in Example 6 was dissolved in 1% acetic acid water (v/v) solution at a concentration of 100 mg/ml for preparative chromatography. A segmented column composed of three PrepPak 40 mm Cartridges (Bondapak C-18 15-20  $\mu$ m, 40x100 mm, Waters) and a guard column (40 x 10 mm) were coupled with a PrepLC 4000 system (Waters). Instrument control, data collection and analysis were undertaken using Millennium Chromatography Manager software v 2.15 (Waters Inc, MA. USA). The sample (20ml) was injected automatically via solvent delivery system. The absorbance profile was monitored at 280 nm using a 2487 Absorbance Detector. Fractions were collected automatically using a Waters Fraction Collector. The column was eluted (50 ml.min<sup>-1</sup>) with a two solvent system: A: water containing 1% acetic acid and B: methanol (100%) (Table 7).

**Table 7. PrepLC 4000 Gradient profile**

<b>Time (min)</b>	<b>%A</b>	<b>%B</b>	<b>Flow rate (ml/min)</b>
0	100	0	50
10	100	0	50
188	55	45	50
198	100	0	50
208	100	0	50
211	100	0	0

All fractions collected were assessed for ACE inhibitory activity. A total of 32.6 g of canola meal Alcalase hydrolysate was fractionated by preparative chromatography and the yield of the most active peptide fraction was 2.8 g (8.5%) with an ACE inhibitory IC<sub>50</sub> of 2.0 µg powder/ml.

5        The active fraction from the preparative C-18 was further purified by Sephasil™ Peptide C18 ST 10/250 column (particle size:12 µm, Pharmacia Biotech, Sweden) coupled with an ÄKTA explorer 10XT system controlled by Unicorn system (Pharmacia Biotech, Sweden). The column was eluted with a two solvent system: A: 20 mM sodium monobasic phosphate buffer solution (0.05% TFA) and B: 20 mM sodium monobasic phosphate (80%  
10 acetonitrile containing 0.05% TFA) at the flow rate of 5 ml/min, after 5 column volume (CV) isocratic elution at 5% B, gradient elution from 5% B- 20% B in 15 CV. The injection volume was 500 µl of a 200 mg/ml solution of the most active preparative chromatography fraction. The absorbance was monitored at 214 nm and fractions were collected. A total of 21  
15 fractions were collected and the most potent fractions were F<sub>13</sub> and F<sub>18</sub>. After repeat injection, 1.30 g of F<sub>13</sub> and 0.85 g of F<sub>18</sub> with ACE inhibitory (IC<sub>50</sub> values of 38.6 and 312.9 µg powder/ml respectively) were obtained. These IC<sub>50</sub>'s were higher than the starting material due to the presence of the non-volatile buffers used in the chromatographic separation.

Each of F<sub>13</sub> and F<sub>18</sub> were re-chromatographed on a Sephasil™ Peptide C<sub>18</sub> ST 10/250  
20 column (particle size:12 µm, Pharmacia Biotech, Sweden) using a two solvent system: A water (containing 0.1% TFA) and B acetonitrile (containing 0.1% TFA) at the gradient of 10% B -30% B in 7 CV at the flow rate of 5 ml/min to de-salt and further purify these active fractions. The major potent fraction peaks were identified as F<sub>13-11</sub> resulting from the chromatography of F<sub>13</sub>, and F<sub>18-5</sub> from the chromatography of F<sub>18</sub>. The yield for F<sub>13-11</sub> was  
25 18.32 mg with an ACE inhibitory IC<sub>50</sub> of 0.44 µg/ml, and for F<sub>18-5</sub> the yield was 4.89 mg with an IC<sub>50</sub> value of 0.37 µg/ml.

Fractions F<sub>13-11</sub> and F<sub>18-5</sub> were further purified (Figure 3) by Sephasil Peptide C<sub>18</sub>.12 ST 4.6/250 column (Pharmacia Biotech, Sweden) using a two solvent system; A: water (containing 0.1% TFA) and B: acetonitrile (containing 0.1% TFA) with a gradient of 5% B -  
30 40% B in 10 CV at a flow rate of 1.5 ml/min. The injection volume was 30 µl of a 25mg/ml solution. The absorbance was monitored at 214 nm and fractions were collected.

The identity of the peptides in the purified single peak fractions F<sub>13-11a</sub> and F<sub>18-5a</sub> from the ST 4.6/250 column were determined by atmospheric pressure chemical ionization (APCi) mass spectrometry using a Quattro LC liquid chromatograph/Mass Spectrometer (Micromass, UK) equipped with APCI probe, Z-Spray interface, separation module and photodiode array detector (Waters Inc. MA, USA). Instrument control and data analysis were performed using the MassLynx software (Micromass, UK). A peptide sample (10ml) was chromatographed on a Symmetry C<sub>18</sub> column (2.0 x 150 mm; 5 mm). The sample was eluted (0.3 ml.min<sup>-1</sup>) with a two solvent system: (A) 0.1% formic acid (FA) in water and (B) 0.1% FA in acetonitrile, with a 5-60 % acetonitrile gradient for the first 10 min, maintained for 2 min at 60% acetonitrile, then returned to 5% acetonitrile for 1 min. This was followed by isocratic elution for 4 min at the constant flow rate of 0.3 ml.min<sup>-1</sup>. Positive and negative ion intensities were recorded from 50 to 500 m/z with a 1.5 sec scan time. The analyzer vacuum was 2.2e<sup>-5</sup> torr. The two most bioactive peptides were identified as Valine-Serine-Valine and Phenylalanine-Leucine (Table 8). Neither of these two amino acid sequences have previously been reported as having ACE inhibitory activity.

**Table 8.** Identity of the two most potent ACE inhibitory peptides from the defatted canola alcalase 2.4 hydrolysate

	F <sub>13-11</sub>	F <sub>18-5</sub>
Sequence	Val-Ser-Val	Phe-Leu
Determined MW	303.21	278.16
Calculated MW	303.18	278.16
IC <sub>50</sub> (µg/ml)	0.44	0.37



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**Table 1**

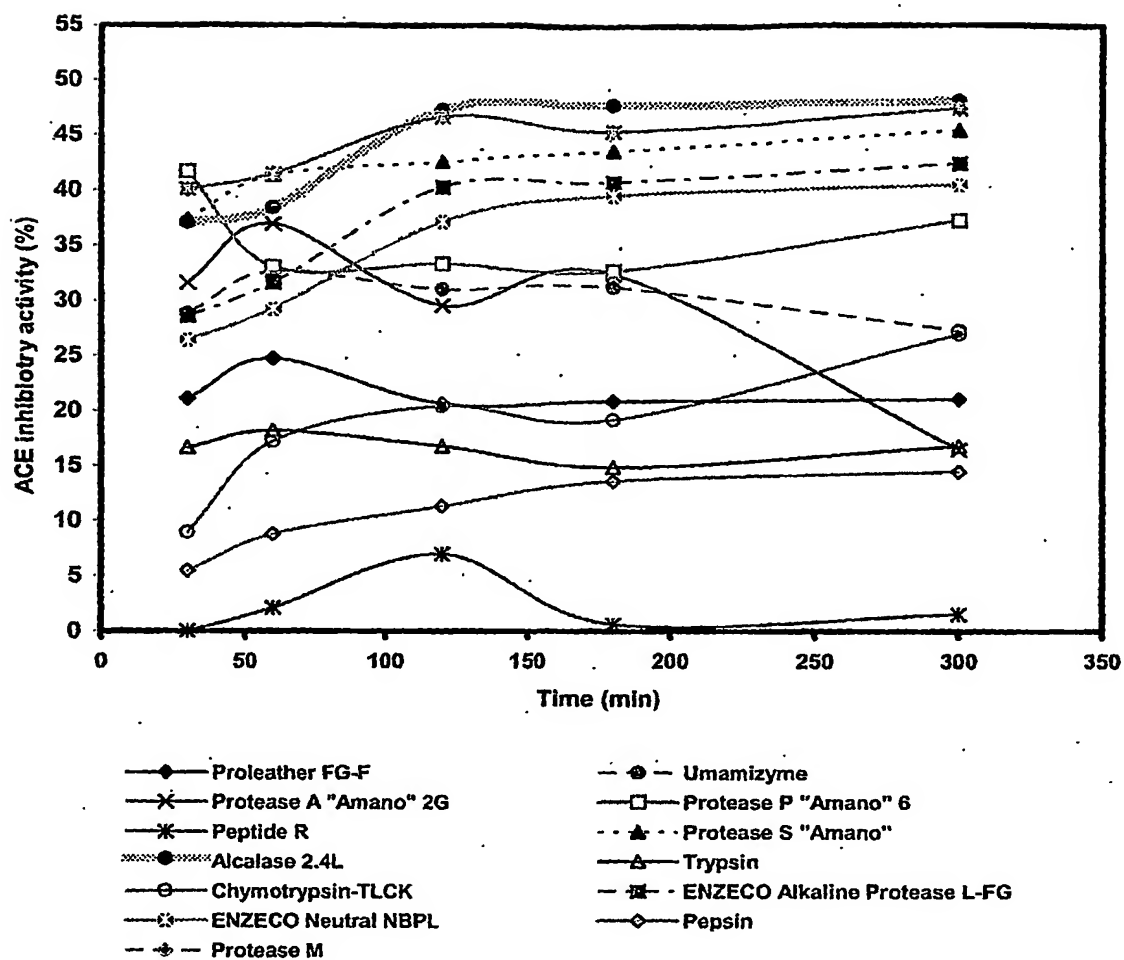
ACE inhibitory IC 50 for alcalase 2.4 digested plant hydrolysates

<i>Raw material</i>	<i>Ethanol extraction</i>	<i>IC50 (<math>\mu</math>g powder/ml)</i>	<i>Protein content (%)</i>
canola meal	yes	41.4	60.4
canola meal	no	68.6	41.7
flax meal	yes	51.4	45.5
flax meal	no	64.3	39.9
pea flour	yes	87.1	63.5
pea flour	no	131.4	59.3
soy meal	yes	126.3	63.8
soy meal	no	118.1	55.3
oat flour	yes	1198.6	8.3
oat flour	no	1044.3	14.2

Table 2

Hydrolysis of canola meal by various proteolytic enzymes

Common Name	Trade Name	pH	T(°C)	Source
Umamizyme	Umamizyme	8	45	Amano Pharmaceutical Co., Ltd. Japan
Protease A	Protease A "Amano" 2G	7	50	Amano Pharmaceutical Co., Ltd. Japan
Protease P	Protease P "Amano" 6	10	60	Amano Pharmaceutical Co., Ltd. Japan
Peptidase R	Peptidase R	7	45	Amano Pharmaceutical Co., Ltd. Japan
Protease M	Protease M "Amano"	4.5	50	Amano Pharmaceutical Co., Ltd. Japan
Protease S	Protease S "Amano"	8	70	Amano Pharmaceutical Co., Ltd. Japan
Proleather FG-F	Proleather FG-F	10	60	Amano Pharmaceutical Co., Ltd. Japan
Alcalase 2.4	Alcalase 2.4L	8	60	Novo Nordisk, North America, Inc., North Carolina.
Alkaline	ENZECO	10	50	Enzyme Development Corporation, New York
Protease	Alkaline Protease L-FG			
Neutral	ENZECO	7	40	Enzyme Development Corporation, New York
Protease	Neutral Protease NBP-L			
Pepsin	porcine stomach mucosa	2	37	Sigma, St. Louis, MO, USA.
Trypsin	Bovine Pancreas	8	37	Worthington Biochemical Corporation, NJ, USA.
Chymotrypsin	Bovine Pancreas	8	25	Worthington Biochemical Corporation, NJ, USA.
Thermolysin	Thermolysin	8	55	Sigma, St. Louis, MO, USA.



**FIGURE 1**

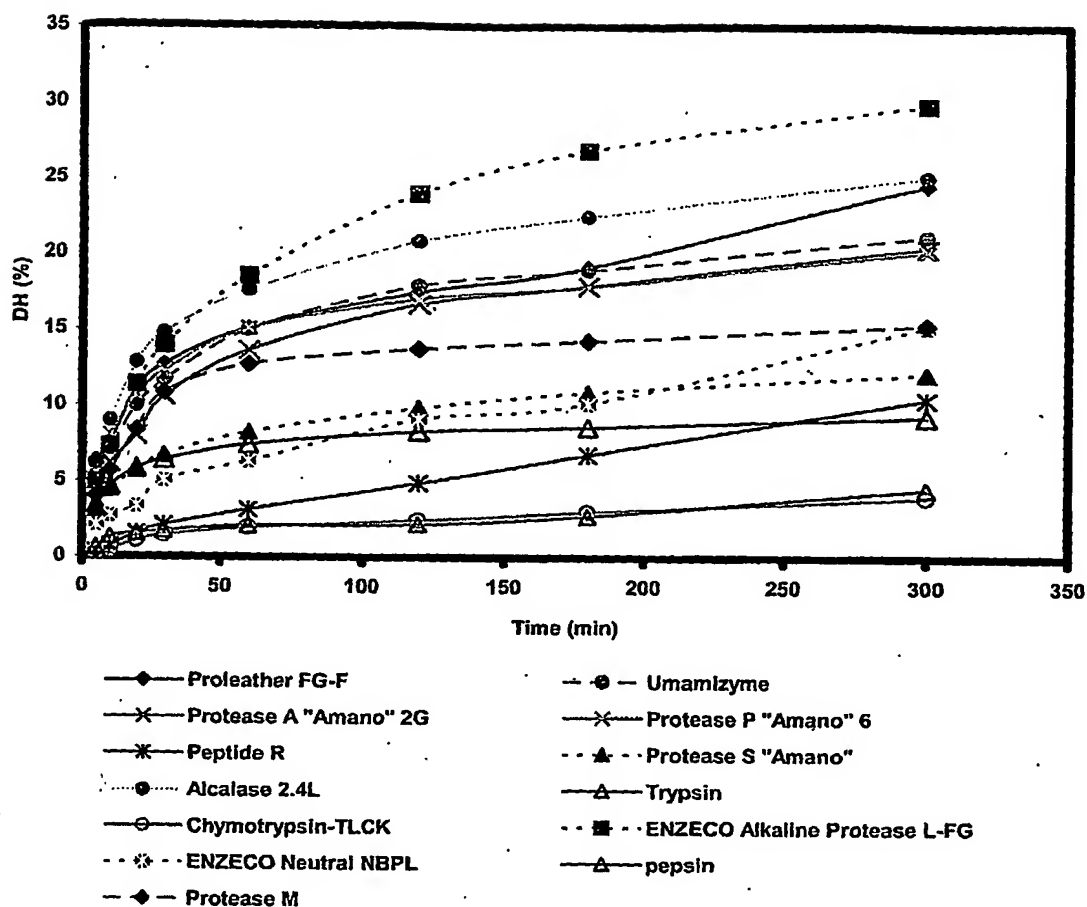
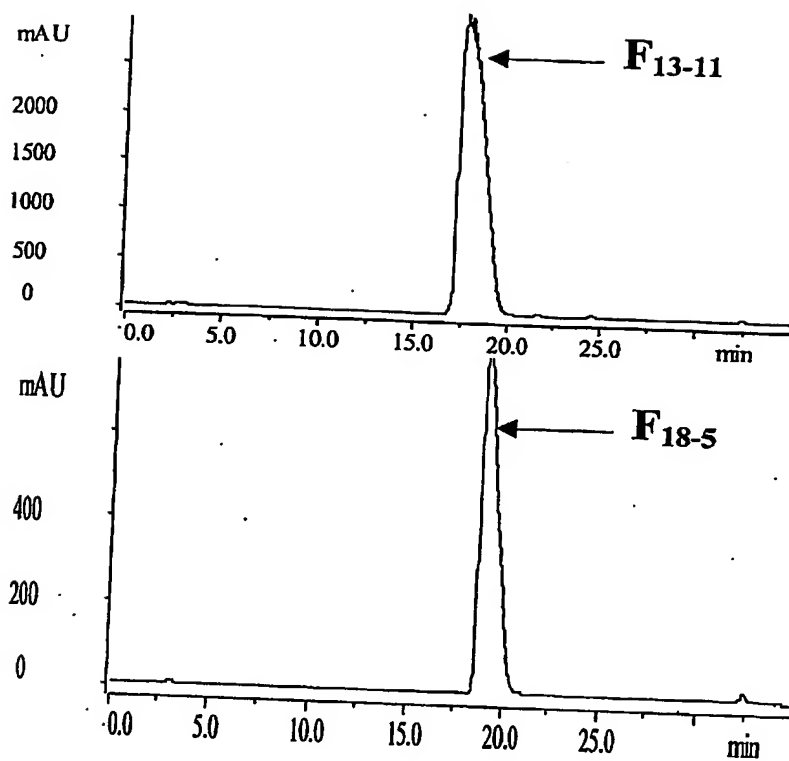


FIGURE 2



**FIGURE 3**

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